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The *hMOF* protein is a chromatin-modifying factor. Chromatin structure plays a critical role in gene expression. Since hMOF has a chromodomain region as well as acetyl transferase activity, its inactivation can influence modification of chromatin during DNA metabolism. The proposed experiments of this grant proposal will determine functions of *hMOF* gene. This will be achieved by generating isogenic cells with and without hMOF function. Both *in vivo* and *in vitro* experiments will be performed to determine the function of hMOF in context with radioresponsiveness and oncogenic transformation. If *hMOF* proves to be involved in the radioresponsiveness and neoplastic transformation, then the clinical implications of this proposal are highly significant. It may, in the future, be prudent to screen each breast cancer patient prior to any final therapeutic decision. This will be accomplished through the use of quantitative RT-PCR and the test results can be obtained within a day. There are several benefits of identifying an individual's normal tissue with loss of hMOF gene expression. First, it will allow us to prospectively identify the sensitive subset of patients. Second, the radiosensitive patients will be taken for an alternative therapy if exist and would be spared a great deal of suffering. Third, it will be possible that once we identify a subset of patients that show a genetic basis of radiation sensitivity, the radiation dose to the remaining breast patients could be increased to be more effective for local tumor control. Fourth, it will provide health professionals a molecular diagnostic approach to predict the suitability of an individual for radiotherapy.

Table of Contents

Cover.....**Page 1**

SF 298.....**Page 2**

Table of Contents.....**Page 3**

Introduction.....**Page 4**

Body.....**Page 4**

Key Research Accomplishments.....**Page 6**

Reportable Outcomes.....**Page 6**

Conclusions.....**Page 6**

References.....**Page 7**

Appendices.....**Page 7**

Introduction:

Most patients with breast cancer tolerate radiotherapy well with only limited acute, reversible adverse effects. However, about 5% of patients experience severe, delayed complications such as skin pigmentation changes, subcutaneous fibrosis, rib fractures, cardiac disease, pulmonary fibrosis, second primary cancer (specifically esophageal squamous-cell carcinoma as well as adenocarcinoma) and other complications, which manifest several years after treatment with ionizing radiation. Epidemiological studies have shown that irradiation of the breast especially among young women, increases the risk for subsequently developing breast cancer. It might thus be expected that genes that are known to influence radiation sensitivity may be associated with the radiotherapy related adverse effects. The human genes that have been found to be responsible for ionizing radiation sensitivity are *ATM* (ataxia telangiectasia mutated), *BRCA1*, *BRCA2, NBS1,* etc. Mutations in *BRCA1* and *BRCA2* contribute to about 15% of familial breast cancer risk and their contribution to sporadic breast cancer is very low. In such cases, genes frequently altered in the general population, e.g., *ATM* may be an important risk factor. However, screening for *ATM* mutations in sporadic breast cancer cases has not revealed the magnitude of involvement of the *ATM* gene expected. Since ATM as well as BRCA1 have been reported to interact with chromatin modifying factors, it is possible that such factors may be involved in the radiation-induced morbidity. Therefore, there is a need for the identification of chromatin modifying factors involved in ionizing radiation sensitivity, genomic instability and carcinogenesis.

Body

Specific Aims:

The goal of this proposal is to understand the mechanisms underlying radiosensitivity. Two specific questions are being addressed in this grant application: (1) Whether hMOF is involved in ionizing radiation (IR) response and; (2) Whether hMOF is involved in pathobiology of the breast cancer. We proposed to complete the following aims: (1) To determine whether mutations in the *hMOF* gene correlate with ionizing radiation sensitivity. (2) To generate MOF knockout mice in order to determine the pathobiology of gene. (3) To determine whether ionizing radiation enhances neoplastic transformations in mouse embryonic fibroblasts of MOF knockout mice. MOF knockout mice will also be examined for spontaneous as well as IR-induced tumor formation.

Studies and Results at the end of project funding:

During the first year, we have addressed the specific aim 1. This specific aim allowed us to determine whether inactivation of hMOF leads to enhanced cell killing by IR treatment. We cloned the full length cDNA as well as fragments of hMOF and expressed in human cell line. We carried out the functional characterization of hMOF and details were published (Gupta et al., 2005. Mol Cell Biol. 25:5292-5305).
During the second year, we have addressed the specific aim 2. This specific aim allowed us to determine the interaction of hMOF with ATM and generate mouse MOF targeting vector for generating mouse knock out mice (Gupta et al., 2005. Mol Cell Biol. 25:5292-5305).
The work completed in subsequent period is summarized below:

Loss of MOF and H4K16 acetylation correlates with early embryonic lethality

MOF is expressed ubiquitously in all mouse tissues. Mouse Mof (*mMof*) gene localizes on chromosome 7 and expresses a protein product of ~58 kD, a similar molecular weight to human MOF protein. To determine the physiological significance of mammalian *MOF* gene, we disrupted the *mMof* gene by homologous recombination in embryonic stem (ES) cells and generated germ line transmitting chimeras.

Heterozygous ($mMof^{+/-}$) intercrosses yielded wild type ($mMof^{+/+}$) and heterozygous ($mMof^{+/-}$) offspring pups but no homozygous *mMof* null mutant pups. The complete absence of homozygous Mof null mutant pups in all litters examined implied that *Mof*-deficiency is embryonic lethal. We then determined the stage of lethality by analyzing embryos from $mMof^{+/-}$ intercrosses at different times postcoitum. In the litters dissected between embryonic day (E8.5) and E10.5, we found approximately 25% empty or necrotic deciduae and all the morphological normal embryos were either wild type or heterozygous. At E8.5, all the normal embryos (wild type and heterozygotes) had gastrulated, while mutant deciduae contained clusters of cells within a small sac or some giant cells only, indicating that *Mof*-deficient embryos are capable of implantation but die prior to the onset of gastrulation.

The observations that no $mMof^{-/-}$ embryos were found at the E8.5 stage of development, led us to investigate MOF protein and acetylation status of H4K16 during early embryogenesis (single cell zygote to preimplantation blastula stages). The entire zygotic cell including the cytoplasm and the chromatin regions demonstrated MOF protein localization, indicating a strong maternal contribution of MOF. Both male and female pronuclei chromatin in the fertilized egg stained equally strong for acetylated histone H4K16 (H4K16ac). Although, all zygotes were positive for MOF as well as H4K16ac staining, about 25% zygotes were null for *mMof* gene. It is interesting to note that H4K16ac mark at the pronucleus stage differs from the histone 3 lysine 9 methylation (H3K9me) and H3K4me modifications, which is markedly stronger on the female than the male pronucleus. Through the first few cleavages, until the 16-cell stage embryo is reached, neither MOF nor H4K16ac immunofluorescence distinguishes $mMof^{-/-}$ embryos from the $mMof^{+/+}$ or $mMof^{+/-}$ embryos. This is most likely due to the abundance of maternal MOF protein present in these cells, which allows for continued development and normal levels of H4K16ac in the chromatin.

Starting at the morula stage, differences between $mMof^{+/+}$ and $mMof^{-/-}$ embryos become apparent, as the maternal MOF diminishes progressively and nuclei specific

MOF signals begin to emerge above the cytoplasmic background. Despite the differences in nuclear levels of MOF in blastomeres of *mMof*$^{+/+}$ and *mMof*$^{-/-}$ embryos, H4K16ac levels remained relatively constant at this stage. In contrast, the maternal MOF protein as well as the H4K16ac levels are barely detectable (if not absent) in *mMof*$^{-/-}$ embryos at the late morula/blastocyst stage of development. Curiously, both the interphase chromatin as well as metaphase chromosomes show comparable H4K16ac levels that are confined to the DAPI stained DNA regions. The deformed nuclear morphology observed by DAPI stain in the *mMof*$^{-/-}$ blastomeres at morula stage and also blastocyst stages could be a precursor of the upcoming catastrophic effects on development.

Very interestingly, some cells of the *mMof*$^{+/+}$ late morula stage express higher levels of MOF as well as higher levels of H4K16ac, a pattern seen among several embryos of this stage. The polarized configuration of these cells and the upcoming differentiation of trophectoderm and Inner Cell Mass (ICM), suggest these cells may be the progenitors of the ICM. This is further substantiated by the observation of differentiated trophoblasts and pluripotent un-differentiated ICM cells in the *mMof*$^{+/+}$ blastocyst. ICM cells have convincingly higher levels of MOF protein and also higher levels of H4K16ac. These observations are consistent with previous results with regard to the higher levels of H4K16ac in embryonic stem cells compared to trophectoderm. The increased acetylation of H4K16 in embryonic stem cells is indicative of the significance of this chromatin modification for proliferation during development. Similar to that observed with H4K16ac, we also examined whether other known chromatin modifications associated with euchromatin increased. Interestingly, on probing for H3K4 trimethylation (H3K4m3), which is associated with a euchromatin conformation, we did not find any preferentially higher level of staining in the nuclei of undifferentiated, pluripotent ICM cells compared to the trophoblasts. The blastocyst and earlier developmental stages displayed uniform and similar levels of H3K4 trimethylation. This observation reveals an important functional attribute of H4K16ac, as a specific histone modification mark in the context of embryonic development. A plurality of chromatin modifications, which achieve relaxed conformation end point, therefore, seem to be providing essential non-redundant and non-overlapping functionalities in different biological scenarios.

Eventually complete depletion of MOF and loss of H4K16ac from a few blastocysts (following Mendelian ratios) was observed. This correlated with the embryos exhibiting a marked delay in developmental progression, sluggish hatching and implantation, proliferation arrest and death. These embryos were identified by genotyping as *mMof* nulls. Notably, depletion of MOF had no effect on histone H3K4m3 levels, an euchromatic marker for active transcription, indicating the lack of cross-talk between these two epigenetic modifications and also an inability of H3K4me3 to propel developmental progression on its own. Retarded progression became more evident as the embryos reached the blastocyst stages.

MOF levels correlate with the frequency of oncogenic transformation

One of the hallmarks of malignant transformation is genomic instability, which promotes a wide range of mutations, including structural and numerical alterations of

chromosomes. In order to gain further insights into the function of mammalian MOF during oncogenesis, we examined cells expressing either mutant or wild type mMof for IR-induced cell killing and oncogenic transformation. Cells expressing ectopic mMof have higher levels of mMof and H4K16ac as compared to cells expressing mutant mMof (ΔmMof). Cells expressing ΔmMof had decreased survival and reduced spontaneous as well as ionizing radiation (IR) -induced oncogenic transformation compared to cells over expressing mMof as determined by foci formation. We further determined the levels of mMof and H4K16ac in normal and tumor mouse tissues. Tumor tissues have relatively higher levels of MOF as well as H4K16ac. Such studies revealed that cells over expressing mMof have higher levels of H4K16ac and increased frequency of transformation. Consistently tumor cells show higher levels of mMof as well as H4K16ac, further supporting an argument about the relationship between the status of H4K16ac and cellular proliferation.

MOF levels correlate with tumor growth

To determine whether hMOF levels influence tumor growth, we used a standard nude mice assay to examine tumor growth and its response to ionizing radiation exposure. Knock down of hMOF in colorectal carcinoma (RKO) cells resulted in the inhibition of cell growth, conversely, over expression of hMOF increased H4K16ac and decreased the population doubling time. RKO cells, with or without over expression of hMOF, were injected into mice; when the tumors reached 8 mm, the mice were irradiated with a single dose of 25 Gy. Untreated tumors arising from RKO cells with hMOF over expression grew rapidly as compared to control cells. Tumors from RKO cell, with and without over expression of hMOF grew at relatively constant rates whereas radiation treatment (single dose of 25 Gy) caused temporary shrinkage of the tumors, followed by regrowth in most of the tumors. Interestingly, tumors from RKO cells with hMOF over expression regrew faster than controls. Two months after irradiation, a single dose of 25 Gy, ~20% of the tumors from control cells were below detectable levels compared to only ~8% tumors from cells over expressing hMOF. An immediate relapse of tumor growth was much more prominent in cells expressing hMOF. These results suggest that hMOF promotes tumor growth.

During the fifth year, we complete the work proposed under task 3.
The global ablation of Mof function in the mouse resulted in early embryonic lethality, we constructed a targeting vector for conditional mutagenesis, which will allow the global and the tissue-specific inactivation of *Mof* studies in future. The details of task 3 has been published recently (Gupta et al., Mol Cell Biol. 2008, 28: 397-409).

Publications:

We have achieved envisaged goals of this grant. During the current funding period 28 papers were published. Each paper contributed directly or indirectly to the over all goals of the proposal.

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f. Project-Generated Resources:

Research supported by this grant resulted in generation of mouse heterozygous for MOF.

Appendix: None